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# Bioengineering bacterial outer membrane vesicles as delivery systems for RNA therapeutics targeted to lung epithelial cytosols.

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## BACKGROUND

### The problem: Inefficient lung cytosol delivery

Lung epithelial cells provide structural integrity, allow gas exchange and enhance ion and fluid transport. Damaged epithelium leads to the development of respiratory diseases, such as chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), cystic fibrosis, and, lately, the 2019 coronavirus disease (COVID-19).

Effective use of macromolecular therapeutics targeting intracellular components of the lung is severely restricted due to poor cytosolic delivery to the respiratory epithelium (Moschos *et al.*, 2017). Outer membrane vesicles (OMVs) are spherical structures (20-250nm) produced by pathogenic and commensal bacteria, when small portions of outer membrane bulge away from the cell, pinch off, and release into the surrounding environment (Momen-Heravi *et al.*, 2018).

OMVs allow bacteria to interact with their environment and, as a result, exert diverse functions such as host cell interaction and the initiation of pathogenesis, survival during stress conditions by toxin sequestration, and regulation of microbial interactions. These functions are afforded through biologically active proteins and nucleic acids naturally encapsulated in these liposome or virus-like structures. Their utility to man is well-established through their scaled bioproduction and application in several commercially successful vaccine preparations.

Recent production of OMVs from BL21 (DE3)-derived, LPS-deficient *Escherichia coli* (Valentine *et al.*, 2016), commercially available as ClearColi® (Mamat *et al.*, 2015), introduced the possibility of pathogen associated molecular pattern-free OMVs, at least in terms of TLR4 activation.

### Our solution: bioengineered microbial vesicles

Overcome cytosolic lung delivery bottlenecks by bioengineering LPS-free, recombinant, biocompatible OMVs biotherapeutics.

## PROJECT OBJECTIVES

- Produce ClearColi® OMVs expressing a proprietary transmembrane protein (TMP) which facilitates active internalisation pathways across respiratory epithelia specifically.
- Produce bioactive OMVs featuring a series of imaging reporter functions.
- Demonstrate TMP-enhanced OMV targeting to respiratory epithelial cells and cytosolic reporter protein delivery.
- Demonstrate functional biotherapeutic compound delivery within respiratory epithelial cells *in vitro* and air-liquid interface culture.

## STUDY DESIGN

- **Stage 1: Clone production**
  - Clone proprietary TMP, far-red fluorescence protein (tdTomato), and N- or C-terminally-tagged TMP in BL21 (DE3) and ClearColi® *E. coli*.
  - Optimise recombinant protein expression.
- **Stage 2: Scalable production of ClearColi® OMVs**
  - Develop scalable OMV production methods.
  - Characterise clone impact on OMV proteome, transcriptome.
- **Stage 3: Evaluate cytosolic access *in cellulo***
  - OMV cell trafficking, RNA-SEQ, and immunomodulation:
    - In submerged cell culture.
    - in Air Liquid Interface (ALI) primary cell culture.
- **Stage 4: Demonstrate efficient cytosolic biotherapeutic delivery**
  - Generate therapeutic RNA and ribonucleoprotein-containing, TMP-labelled OMVs.
  - Quantify pharmacological activity in translationally relevant cell culture systems.

## REFERENCES

1. Schwechheimer C, Kuehn MJ. (2015). Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol. 13 (10), p605-19. 2. Anand D. and Chaudhuri (2016). Bacterial outer membrane vesicles: New insights and applications. Molecular Membrane Biology. 33 (6-8), p125-137. 3. Klimentova J. and Stulik J. (2015). Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. Microbiological Research. 170(1) p1-9. 4. Moschos SA, Usher L., Lindsay MA (2017) Clinical potential of oligonucleotide-based therapeutics in the respiratory system. Pharmacology & Therapeutics 169(1) p83-103. 5. Momen-Heravi F., Getting S.J. and Moschos S.A. (2018) Extracellular Vesicles and Their Nucleic Acids for Biomarker Discovery, Pharmacol. Ther. p170-187. 6. Mamat U, Wilke K, Bramhil D. et al. (2015) Detoxifying *Escherichia coli* for endotoxin free production of recombinant proteins, Microb Cell Fact. 14 (57) 1-15. 7. Valentine J., Chen L., Perregaux E.C. et al., (2016) Cell Chem Biol., 23 (6), p 655-665

## CLONE PRODUCTION

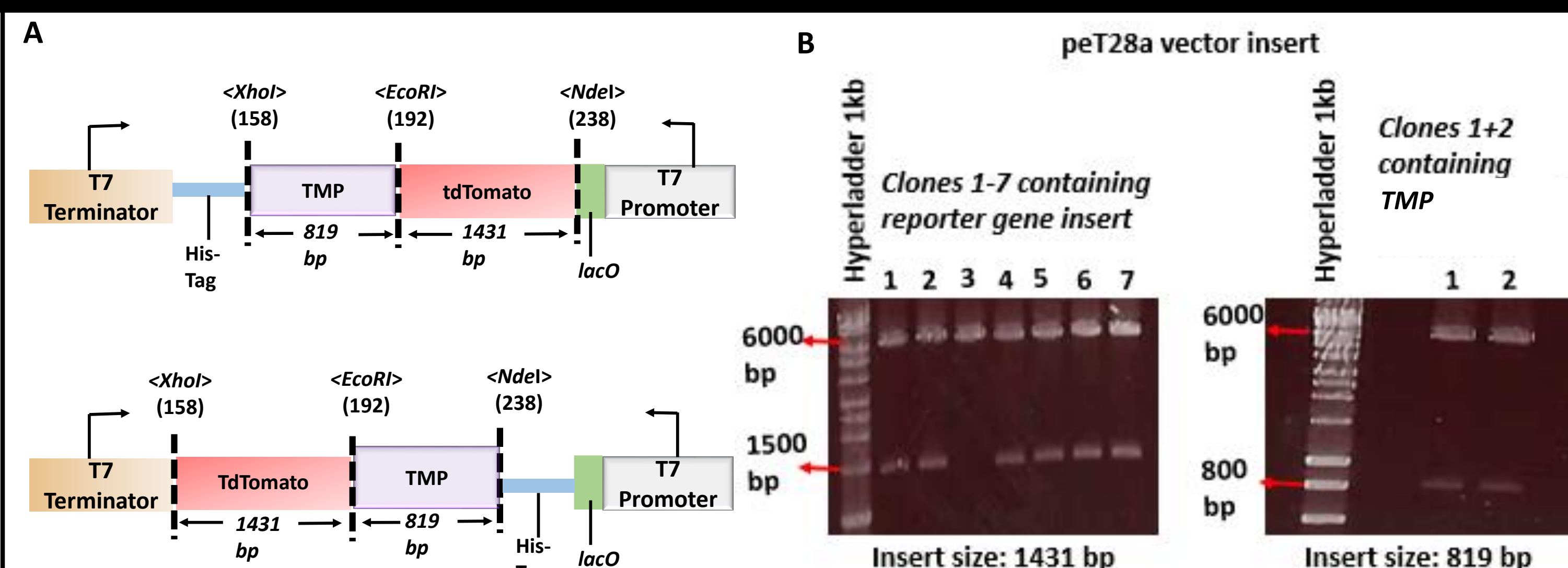


Figure 1. TMP and reporter-expressing ClearColi® clone generation. (A) Construct cloning methodology and (B) Gel electrophoresis to validate the ligation into pET-28a in N- and C-terminal fashion relative to the transmembrane (TMP) protein.

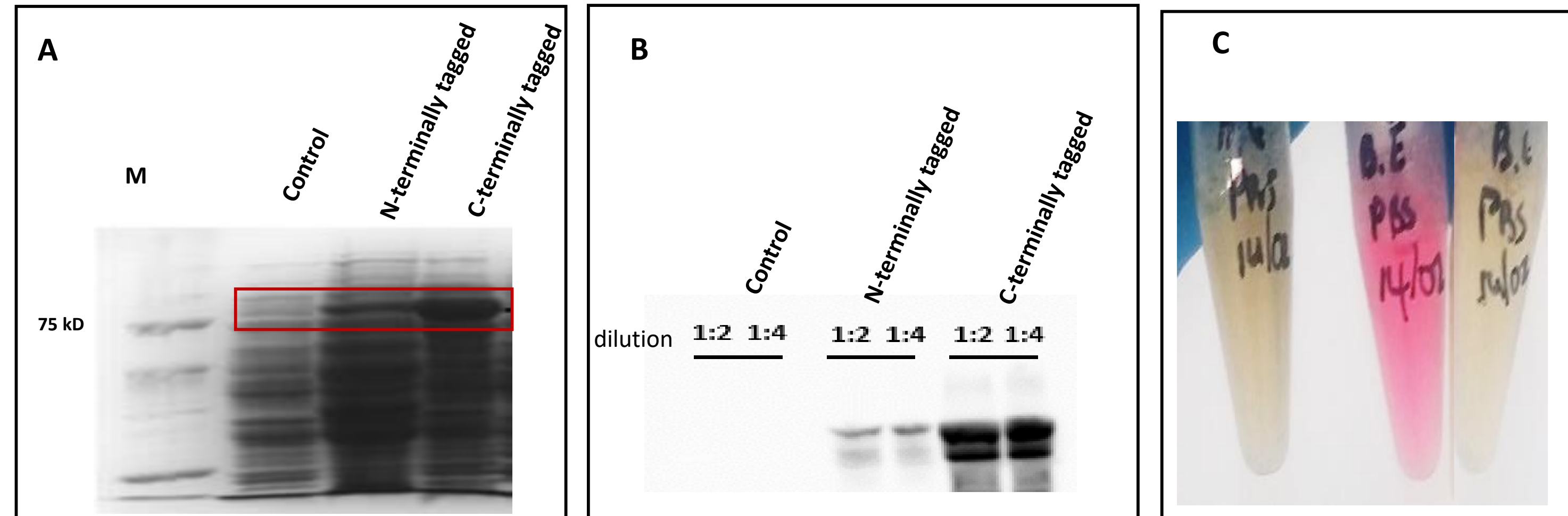


Figure 2. Optimised protein expression. (A) N- and C-terminally tagged fusion constructs were demonstrated by DS-PAGE analysis and (B) Western immunoblotting. Strong band presentation indicates the expression of fusion protein in both orientations, (C) which is visible in the OMV fraction only in N-terminally tagged TMP preps.

## RECOMBINANT TMP-OMVs

- Multiple methodologies have been proposed for OMV bioproduction (Schwechheimer and Kuehn (2015), Klimentova and Stulik (2015), Anand and Chaudhuri (2016).
- Tangential Flow Filtration and Ultracentrifugation return higher purity, scalable, and reproducible OMV preps.

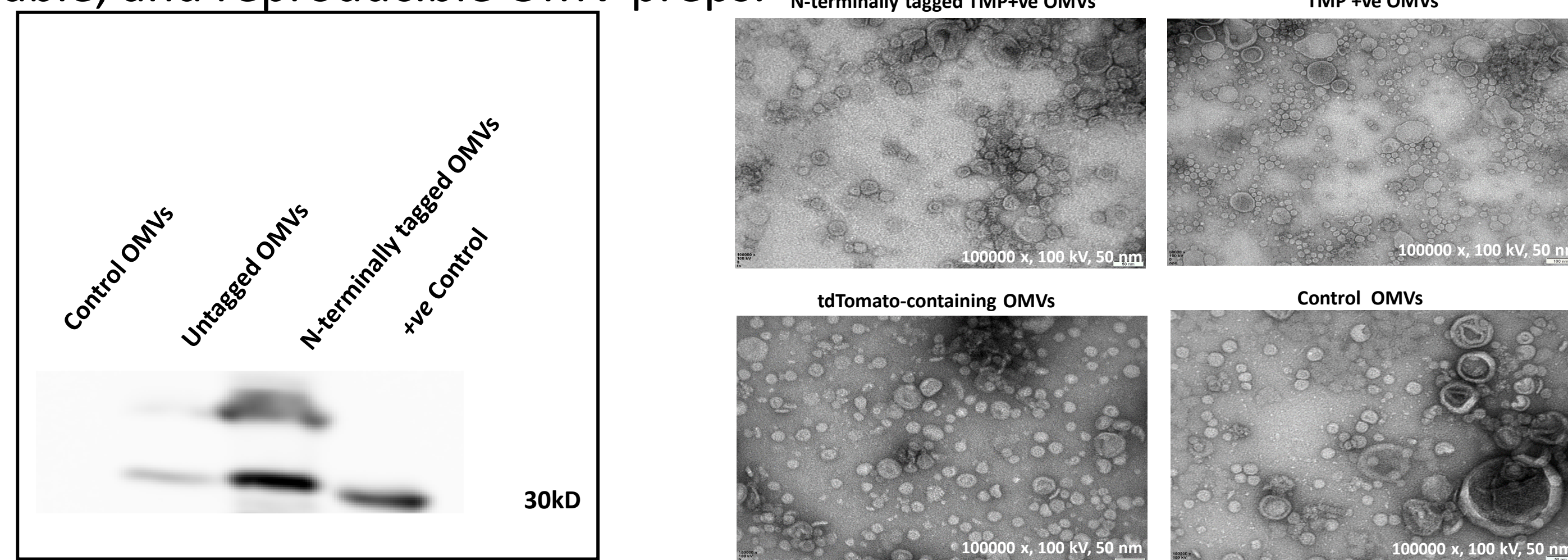


Figure 3: N-terminally tagged TMP efficiently delivers protein cargo to OMVs. Western blotting for the 30 kDa TMP in OMVs succeeds only by N-terminal TMP tagging, simultaneously enhancing OMV transgene loading relative to total protein content.

Figure 4: TEM analysis of bioengineered OMVs. Magnification at 100,000 x, scale bar 50nm, 100 kV. OMV samples were diluted in PBS.

## PROTEOMIC RECOMBINANT OMV ANALYSIS

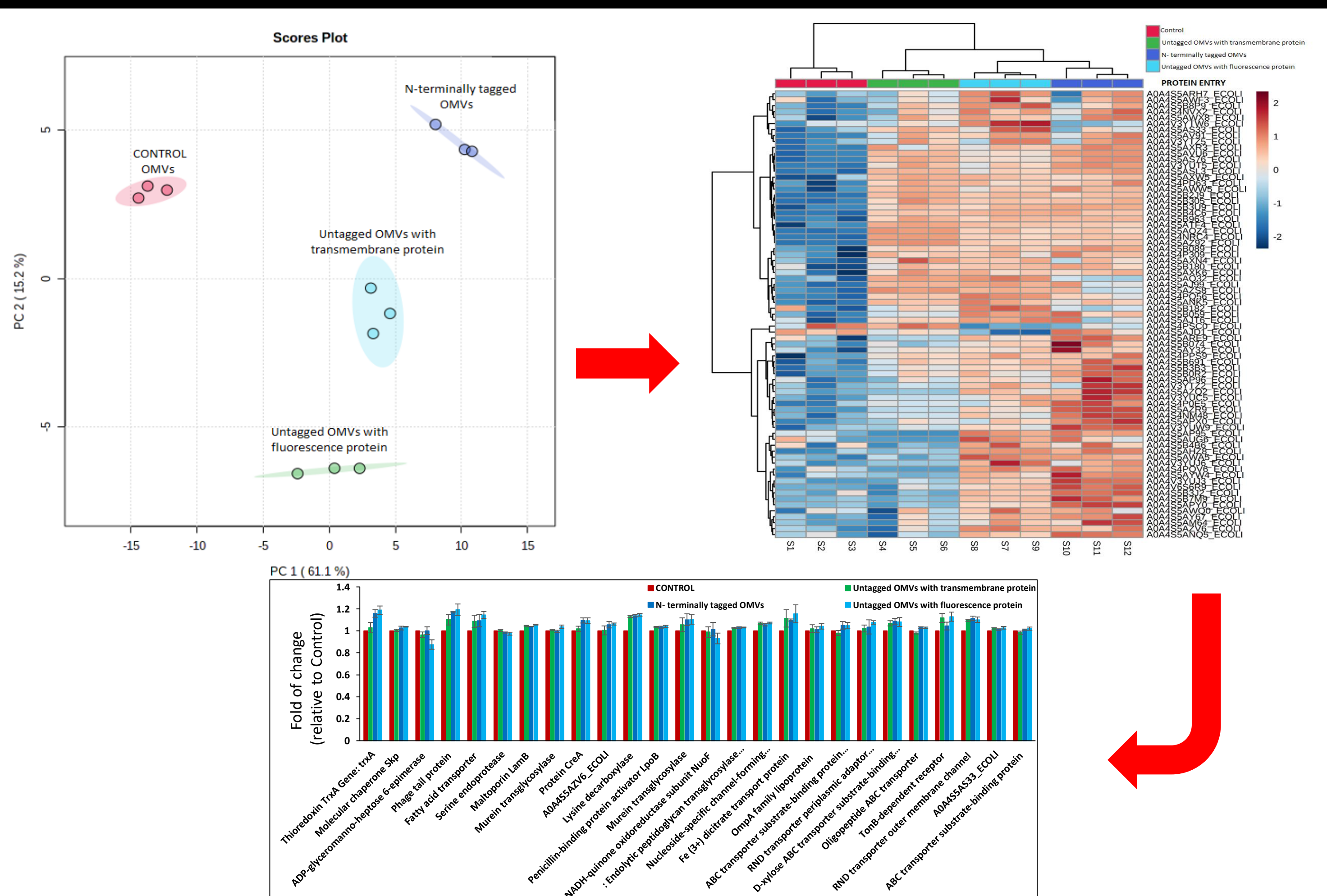


Figure 5: Highly consistent recombinant OMV bioproduction. Three independent biological OMV preps were subjected to proteomic profiling to return 1100 proteins after known contaminant removal demonstrating (A) highly consistent between-replicate prep consistency by unsupervised PCA after log<sub>2</sub> transformation and any missing value imputation prior to visualization. The relative levels of the top 50 discriminates responsible for prep classification are shown by heatmap (B) vs Uniprot protein ID. Statistically significant changes of the top 25 to discriminate marker proteins compared to the Control OMV prep are shown highlighted (C) underscoring the unexpected level of recombinant OMV proteomic diversity resulting through single recombinant protein production, validating the need for comprehensive OMV profiling prior to biological use.

## CONCLUSIONS & FUTURE PLANS

- Eukaryotic receptor ligand TMPs can be ectopically expressed in TLR4-evading ClearColi® bacteria.
- N-terminal tagging enhances TMP loading onto OMV membranes but substantially alters OMV proteome profile.
- Validation of TMP<sup>+</sup> ClearColi® OMV loading and internalization by lung epithelial cells may unlock oligonucleotide, ribonucleoprotein or recombinant/mRNA delivery for chronic, hereditary, and infectious lung disease such as IPF, COPD, cystic fibrosis, and COVID-19.